AWARD NUMBER: W81XWH-14-1-0138

TITLE: Therapeutic Inhibitors of LIN28/let-7 Pathway in Ovarian Cancer

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Houston, TX 77030-5400

REPORT DATE: September 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
September 2015	Annual	30 Aug 2014 – 29 Aug 2015

4. TITLE AND SUBTITLE
Therapeutic Inhibitors of LIN28/let-7 Pathway in Ovarian Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0138

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

There are two major goals of this research project: 1) To define how specific TUTases function in ovarian cancer cells that are either LIN28B-positive or LIN28A/LIN28B-double negative, 2) To perform a pilot small molecule screen in LIN28-positive ovarian cancer cells to identify compounds that restore expression of the tumor suppressor let-7 microRNA family in LIN28-positive ovarian cancer cells. Mechanistically, we envision that these molecules would inhibit LIN28 and its interacting TUTase(s). During this reporting period, we have generated loss-of-function cell lines with reduced TUTase expression singly or in combination, using an RNAi approach. These cells have been analyzed with the respect to cell proliferation and migration. Also, high quality RNA was isolated from multiple loss-of-function cell lines to perform global microRNA profiling to determine if LIN28B requires a TUTase(s) to repress let-7 microRNA biogenesis and if TUTases regulate microRNA expression in the absence of LIN28A/LIN28B expression. In addition, we have generated numerous stable cell lines with distinct let-7 reporters for use in our pilot screen for both LIN28A-positive (OVK-18 and Igrov-1) and LIN28B positive cell lines (TOV-112D).

15. SUBJECT TERMS
Ovarian Cancer, LIN28, LIN28A, LIN28B, let-7, TUTase, 3' RNA uridylation, ZCCHC11, ZCCHC6, Proto-oncogene, Cell-based Small Molecule Screen, Dual Luciferase Reporter

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	16	**19b. TELEPHONE NUMBER** *(include area code)*

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1. **INTRODUCTION:**

Recent and provocative evidence suggests a novel form of post-transcriptional gene regulation involving 3' RNA uridylation mediated by Terminal Uridylyl Transferases (TUTases) as a critical driver of tumorigenesis. Specifically, dysregulated TUTase activity alone and in concert with the onco-fetal LIN28/let-7 pathway are hallmarks of poor prognosis in multiple cancer types, including ovarian cancer. The LIN28 family of RNA binding proteins consists of two related proto-oncogenes, LIN28A and LIN28B, where each protein is able to block the biogenesis and function of the tumor suppressor let-7 microRNA family. LIN28A recruits the TUTase ZCCHC11 to block let-7 maturation, while the TUTase dependence of LIN28B remains unclear. Silent in almost all adult cells, the LIN28/let-7 pathway is implicated in ovarian cancer predisposition by genome wide association studies1 and in ovarian cancer stem cell biology. LIN28 expression correlates with poor ovarian cancer prognosis, consistent with its cancer role in conferring resistance to ionizing radiation and several chemotherapies such as Taxanes and platinum-based drugs. We hypothesize that LIN28 and/or its interacting TUTase are viable molecular targets for ovarian cancer therapies as these proteins are co-opted by malignancies to inactivate the tumor suppressor let-7 microRNA family. Testing this hypothesis is essential to define molecular targets for future therapeutic intervention, including specific TUTases. The proposed research addresses the open question of how TUTases contribute to ovarian cancer in both LIN28B-positive and LIN28A/B-negative ovarian cancer cells. Furthermore, this research seeks to identify LIN28 and/or TUTase inhibitors as a novel therapy through a pilot screen in LIN28-positive ovarian cancer cells.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Ovarian Cancer
LIN28
LIN28A
LIN28B
let-7
TUTase
3’ RNA uridylation
ZCCHC11
ZCCHC6
Proto-oncogene
Cell-based Small Molecule Screen
Dual Luciferase Reporter

3. ACCOMPLISHMENTS:

Major Goals of the Project

The proposed research addresses the open question of how TUTases contribute to ovarian cancer in both LIN28B-positive and LIN28A/B-negative ovarian cancer cells. Furthermore, this research seeks to identify LIN28 and/or TUTase small molecule inhibitors that restore expression of the tumor suppressor let-7 microRNA family through a pilot screen in LIN28-positive ovarian cancer cells. The timeline from the approved SOW is shown below and is annotated with each specific task and completion percentage. For tasks that are spread over year 1 and year 2 time periods, the completion percentage refers to the entire task.

AIM 1	YEAR 1	YEAR 2	Task	Completion
Acquisition of Human Ovarian Cancer Cell Lines			1	100%
Generate and Validate TUTase and LIN28 Loss-of-Function Human Ovarian Cancer Cell Lines			2/3	90%
Identify TUTase dependence of LIN28B			5	50%
Determine Oncogenic Phenotypes Affected by TUTase Loss in Ovarian Cancer Cells			4	20%

AIM 2	YEAR 1	YEAR 2	Task	Completion
Optimize Reporter Screens			6A	100%
Pilot Small Molecule Screen			6B	0%
Validation of Candidate Compounds			6C/6D	0%
Determine Mode of Action of Compounds			6E	0%

Task 1. Acquisition of Human Ovarian Cancer Cell Lines (All Specific Aims, Months 1-2) 100% completed
All the required human ovarian cancer cell lines have been acquired.

Task 2/Task 3. Generation and Validation of TUTase and LIN28 Loss-of-Function Human Ovarian Cancer Cell Lines. (Months 1-14) 90% Completed
Loss-of-function stable cell lines have been generated using multiple shRNAs targeting our genes of interest. In addition, we have employed two additional techniques to generate loss of function lines (siRNA and the CrispR/Cas9 system).

Task 4. Determine oncogenic properties associated with TUTase and LIN28B loss in ovarian cancer cell lines (Specific Aim 1, Months 9-24) : 20% Completed
4a. Cell proliferation (Months 9-24) 50% Completed
4b. Apoptosis (Months 9-24) 0% Completed
4c. Migration (Months 9-24) 50% Completed
4d. Radiosensitivity (Months 9-24) 0% Completed

Task 5. Global microRNA analyses of control and knockdown samples (Specific Aim 1, Months 6-20) 50% Completed
5a. Perform control and knockdown experiments in model ovarian cancer cell lines (Months 6-12) 100% Completed
5b. Isolate total, high quality RNA (Months 6-12) 100% Completed
5c. Determine TUTase dependence of LIN28B-mediated let-7 repression by measuring mature microRNA levels by RT-qPCR in control and knockdown samples (Months 9-18) 0% Completed
5d. Global microRNA profiling by Exiqon qPCR Arrays (UTHSC-H Quantitative Genomic/Microarray Core) (Months 9-20) 0% Completed

Task 6. Small molecule screen for modifiers of the LIN28B/let-7 pathway (Specific Aim 2, Months 6-24) 20% Completed

6a. Establishment and validation of cell-based reporter to monitor the LIN28B/let-7 pathway (Specific Aim 2A, Months 6-10) 100% Completed

6b. Pilot screen for small molecule inhibitors of LIN28B/TUTiLB/let7 pathway (Performed by GCC John Dunn Screening Core, Specific Aim 2B, Months 10-18) 0% Completed

6c. Validation of candidate compounds (Specific Aim 2C, Months 13-20) 0% Completed (Year 2 task)

6d. Global microRNA profiling of cells treated with candidate compounds and controls by Exiqon qPCR Arrays (UTHSC-H Quantitative Genomic/Microarray Core) (Months 15-22) 0% Completed (Year 2 task)

6e. Experimental exploration of mode of action (Specific Aim 2C, Months 16-24) 0% Completed (Year 2 task)

Accomplishments for These Major Goals

Task 1. Acquisition of Human Ovarian Cancer Cell Lines (All Specific Aims, Months 1-2) 100% completed

All the required human ovarian cancer cell lines have been acquired. Several human ovarian cancer cell lines were obtained from a different source than initially listed in the SOW. This change occurred for two reasons: 1) cell line availability issues at Creative Bioarrays and 2) the temporary moratorium on providing cell lines from DTP/DCTD Tumor Repository of the NCI at the beginning of the funding period. Notably, OVK-18 was obtained from RIKEN Cell Bank, SK-OV-3 was obtained from ATCC, IGROV-1 was a kind gift of Dr. Anil Sood, and COLO-704 was purchased from DSMZ-German Collection of Microorganisms and Cell Cultures. To verify LIN28 expression, we have performed Western blot analysis to interrogate LIN28A and LIN28B expression. Representative Westerns are shown in Figure 1. These results confirm the LIN28 expression status for our selected ovarian cancer cell lines.

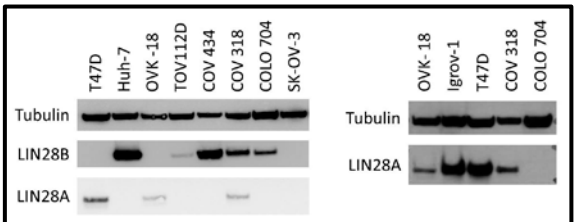

Figure 1. Western Blot Analysis of LIN28 Expression in Human Cancer Cell Lines. A panel of human ovarian cancer cell lines were interrogated for expression with antibodies corresponding to the listed gene. T47D is a control LIN28A-positive breast cancer line, while Huh-7 is a control LIN28B-positive liver cancer cell line. These results are consistent with LIN28 expression in the CCLE database.

Task 2/Task 3. Generation and Validation of TUTase and LIN28 Loss-of-Function Human Ovarian Cancer Cell Lines. 90% Completed

In our application, we proposed to use Cre-mediated recombination to generate shRNA knockdown cell lines for TUTases and LIN28. Unfortunately, we have not succeeded in generating the shRNA lines using this particular technique. Therefore, we used a standard lentiviral shRNA approach as we described previously to create the loss-of-function cell lines. This alternate method was explicitly included in our OCRP Pilot Award application [Aim 1A "Expected Results and Potential Caveats" Section (Page 4/Lines 22-25)]. Specifically, it was written: "If the proven doxycycline system for shRNA expression does not work for unexpected reasons, we will use standard lentiviral shRNA infections followed by puromycin selection to achieve knockdowns as we described previously[12]." A representative Western is shown in Figure 2. In addition to shRNA, we have utilized siRNAs as another loss-of-function RNAi technique. In general, this method yielded more consistent results and more efficient knockdowns than the shRNA method. Figure 3 shows representative siRNA-mediated knockdowns in two ovarian cancer cell lines.

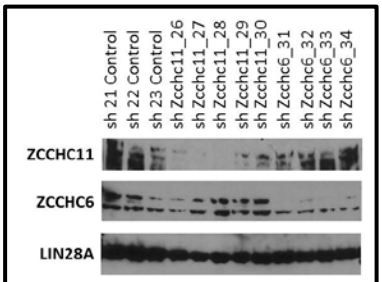

Figure 2. Western of Control and TUTase Stable shRNA Knockdowns. LIN28A serves as the loading control.

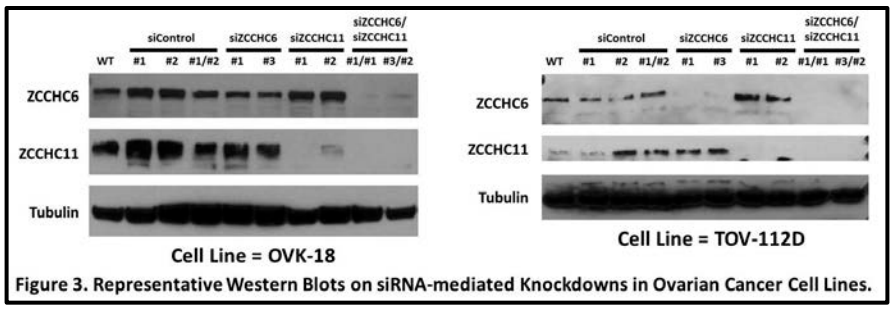

Figure 3. Representative Western Blots on siRNA-mediated Knockdowns in Ovarian Cancer Cell Lines.

As an alternate technique to generate stable loss-of-function cell lines to complement our already generated shRNA lines, we are developing handson experience with CrispR/Cas9 technology to generate stable cell lines where our genes of interest will be inactivated. The advantage of the CrispR/Cas9 method is that stable lines can be established that have one or more alleles mutated, resulting in loss-of-function. As such, it is possible to select clonally stable cell lines that are null for expression of our genes of interest in comparison to our stable shRNA lines where the expression of the targeted genes is reduced but not absent. As proof-of-principle, we have performed transient transfection with a dual Cas9/gRNA plasmid and have selected clones using limited dilution without selection. The gRNAs were designed to target exon 2 of LIN28A and LIN28B. For ZCCHC6 and ZCCHC11, the gRNA was designed against the exon containing the first two aspartates of the catalytic triad. Using this method, we have generated clonal cell lines where 1 or 2 alleles have been mutated for LIN28B. ZCCHC11, or ZCCHC6. Of note, the cell line where we initially tested this technique was TOV-112D that is triploid at the genome level for both LIN28B and ZCCHC6. As Figure 4 illustrates, our results show that our gRNAs are working to direct Cas9-mediated double strand breaks where non-homologous end joining (NHEJ) leads to insertions/deletions. Although a line may lose all wild-type alleles, validation by western is important to confirm that the mutations abolishes protein expression as there will be cases where for example a small deletion does not change the open reading frame. To optimize further, we plan on introducing both Cas9 and specific gRNAs for our genes of interest using lentiviral infection with antibiotic selection to improve the rate of generating null lines. The resulting cell lines will be validated as null at the protein level by Western. To this end, we have tested several lentiviral packaging mixes with the Zhang's Lenti-Cas9-Blasticidin construct. As Figure 5 shows, we have been able to establish stable ovarian cancer cell lines that express Cas9 as determined by Western and immunohistochemistry.

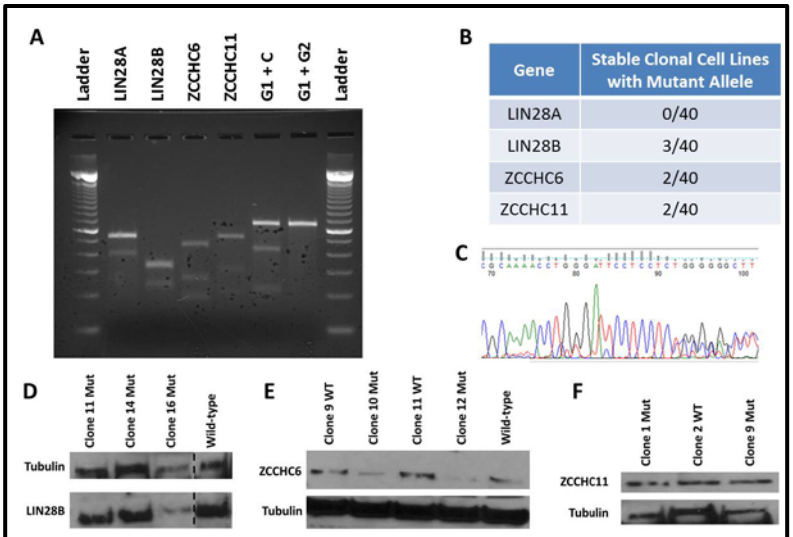

Figure 4. CrispR/Cas9-Directed Mutation of LIN28 and TUTases. TOV-112D cells were transiently transfected with a dual Cas9 /gRNA plasmid where the gRNA was specific for the genes indicated. A) Surveyor assays reveal a significant proportion of the total cell population is characterized by indels at the corresponding genomic loci. Specifically, genomic DNA was isolated ~1.5 weeks post-transfection. For the surveyor assay, the top band is the wild-type allele, while the lower bands represent the mutant allele. Of note, G1 + C was a positive control for the surveyor assay, while G1 + G2 was a negative control. B) Table shows the frequency of independent clonal lines generated by limited dilution from the transient transfections that harbor at least one mutant allele. C) Representative sequencing of PCR amplicon corresponding to gRNA/Cas9-targeted genomic DNA with a WT and indel allele from a clonal line (mutant LIN28B). D-F) Westerns are shown for clonal mutant and wild-type lines for LIN28B, ZCCHC6, and ZCCHC11, respectively. Of note, TOV-112D are triploid at the genome level for both LIN28B and ZCCHC6 due to chromosomal gain.

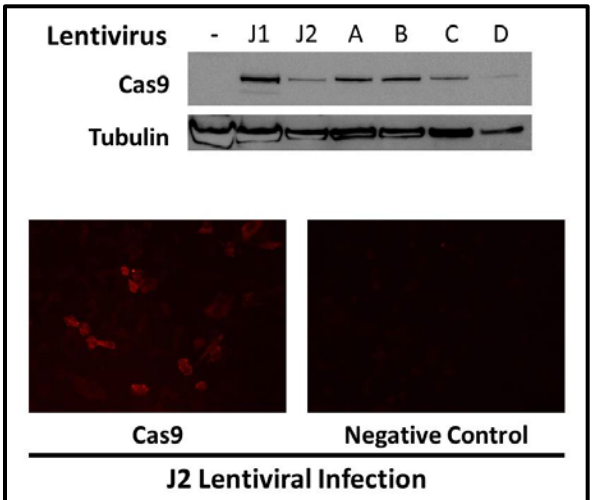

Figure 5. Verification of Cas9 Expressing Ovarian Cancer Cell Lines. Six distinct lentiviral preparations using different combinations of Cas9 constructs and packaging plasmids were used for infection and then selected with blasticidin. Top panel. Western blot reveals that all transduced lines are positive for Cas9 by Western. Bottom panel. Immunohistochemistry was done on cells derived from the J2 infection revealing predominant Cas9 staining in the cytoplasm which is consistent with its subcellular localization in the absence of gRNA.

Task 4. Determine oncogenic properties associated with TUTase and LIN28B loss in ovarian cancer cell lines (Specific Aim 1, Months 9-24) : 20% Completed

To date, we have interrogated how cellular proliferation and migration is affected by TUTase loss in the LIN28B-positive TOV-112D) and the LIN28A/B-double negative (SK-OV-3) ovarian cancer cell lines. As Figure 6 depicts, there are no compelling differences in proliferation upon ZCCHC6 or ZCCHC11 loss singly or in combination as determined by the MTT assays at 72 hours for the TOV-112D cell line. In contrast ZCCHC11 loss impaired cellular proliferation in the SK-OV-3 cells. Our migration assays reveal that loss of either TUTase ZCCHC6 and ZCCHC11 impaired dramatically migration in both interrogated cell lines. As described in our initial application, we will expand this work to additional ovarian cancer lines.

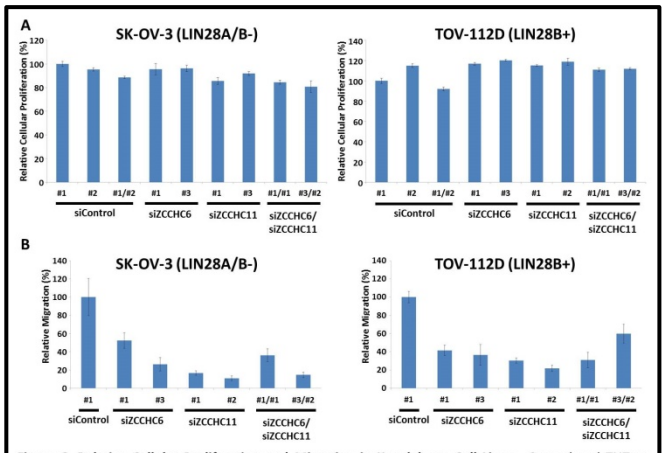

Figure 6. Relative Cellular Proliferation and Migration in Knockdown Cell Lines. Control and TUTase knockdowns were performed on the indicated ovarian cancer cell lines. Data was normalized relative to the siControl #1. A) Cellular proliferation was assessed by the MTT assay at 72 hours post-knockdown in the indicated cell lines. B) Transwell assays were used to measure migration. Specifically, cells were reseeded at the same cell density 72 hours post-transfection in transwells and the number of migratng cells measured at 88 hours posit-transfection.

Task 5. Global microRNA analyses of control and knockdown samples (Specific Aim 1, Months 6-20) 50% Completed

Using RNAi, we have performed control and knockdown experiments in model ovarian cancer cell lines and verified the extent of protein loss by Western as described in detail above in Task 2/Task 3 Accomplishment Section. In addition, we have isolated high quality RNA from these knockdowns (Figure 7) in order to analyze individual and global microRNA expression. To date, we have high quality RNA from a handful of ovarian cancer cell lines such as TOV-112D, COV-434, SK-OV-3, OVK-18, and Igrov-1.

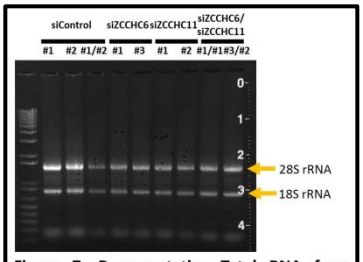

Figure 7. Representative Total RNA from Control and TUTase Knockdowns (SK-OV-3).

Task 6. Small molecule screen for modifiers of the LIN28B/let-7 pathway (Specific Aim 2, Months 6-24) 20% Completed

For this task, we have generated let-7 reporters based on two distinct systems. The first is a dual luciferase reporter when the expression of nanoluc luciferase is regulated by let-7 levels while the second system is based on let-7 regulation of a Thymidine Kinase-Zeocin resistance fusion gene whose expression confers differential sensitivity to ganciclovir. In each case, four different constructs have been generated that are based on the 3' UTR of TRIM71, a well-characterized let-7 regulated gene. These DNA fragments were cloned immediately downstream of the end of the relevant open reading frame (i.e, nanoluc or TK-ZeoR). Specifically, the constructs contain: 1) wild-type TRIM71 with its two imperfect let-7 binding sites, 2) TRIM71 with both imperfect let-7 binding sites deleted (negative control), 3) TRIM71 with one "perfect" let-7 binding site, and 4) TRIM71 with two "perfect" let-7 binding sites. As an example, Figure 8 demonstrates our work with TK-ZeoR-based reporter in the TOV-112D cell line. The cell based-reporters with both perfect and imperfect let-7 binding sites show dramatically increased survival in ganciclovir at 2uM concentration in relation to the negative control. Overall, stable cell lines for each construct have been established for three ovarian cancer cell lines: LIN28A-positive Igrov1 and OVK-18 and LIN28B-positive TOV-112D.

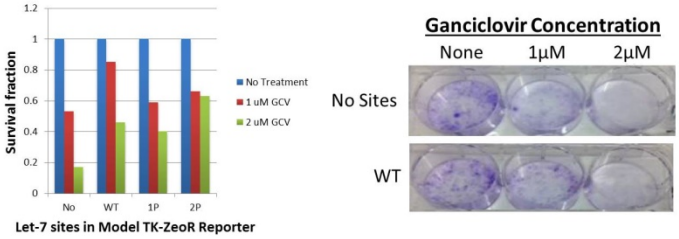

Figure 8. Validation of Cell-Based Let-7 Reporter. Stable cell lines were established for each of the let-7 reporters containing TK-ZeoR as well as a negative control with no let-7 sites. WT contains two imperfect sites, 1P has one "perfect: let- site, while 2P has two perfect let-7 sites. In the presence of ganciclovir, expression on the TK-ZeoR leads to cell death that is rescued when let-7 sites are present in the 3' UTR of the TK-ZeoR.

Training and Professional Development Provided by the Project

This OCRP Pilot Award includes Dr. Julien Balzeau as a nested TEAL Postdoctoral Researcher. During the first year of the award period, he has met on a regular basis (almost daily during the work week) with the Principal Investigator to discuss the ongoing research, experimental design, problems encountered and their solutions, and professional development. In addition, he has been actively involved in the Postdoctoral Certificate Training Program that includes numerous lectures on topics such as career development, responsible research conduct, and teaching skills. He frequently participates in the Houston RNA Club hosted by Baylor College of Medicine. This weekly series during the academic year includes both data presentations of local Houston RNA research as well as a journal club of recent, high impact RNA papers. Julien also expanded his knowledge of ovarian cancer research in several key ways. Specifically, he attended the 2015 Annual Meeting on Women's Cancer held in Chicago from March 28-31, 2015. This meeting provided a unique opportunity to learn more about basic and clinical ovarian cancer research. Of particular note, Julien as well as the rest of the Hagan research team have attended Dr.Anil Sood's weekly lab meetings over the past year at MD Anderson Cancer Center. Dr. Sood is an outstanding physician scientist and leading ovarian cancer researcher. Overall, Julien has presented two journal clubs and two data presentations.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

In the second and final year of this award, we plan to complete all the tasks as outlined in the SOW. Specifically:

Task 1. Acquisition of Human Ovarian Cancer Cell Lines (All Specific Aims)
We will continue to verify routinely all of our acquired human ovarian cancer cell lines to make certain that we are using the correct cells for our experiments.

Task 2/Task 3. Generation and Validation of TUTase and LIN28 Loss-of-Function Human Ovarian Cancer Cell Lines. (Specific Aim 1)
We have already generated loss-of-function ovarian cancer cell lines using two distinct RNAi methods, shRNA and siRNA. We plan to explore the feasibility of using the CrispR/Cas9 system to generate knockout lines that have the lost expression of our genes of interest in their entirety instead of our generated knockdown lines where expression is reduced.

Task 4. Determine oncogenic properties associated with TUTase and LIN28B loss in ovarian cancer cell lines (Specific Aim 1).
Using the loss-of-function lines from Task 2/Task 3, we will interrogate how TUTase loss affects cell proliferation, migration, apoptosis, and lastly, survival upon exposure to ionizing radiation in both LIN28B-positive and LIN28A/LIN28B-positive ovarian cancer cells. During year 1, we investigated cellular proliferation and migration for TOV-112D and SK-OV-3 cells. We will expand this to additional ovarian cancer cell lines as well as all the aforementioned assays.

Task 5. Global microRNA analyses of control and knockdown samples (Specific Aim 1)
Using the high quality RNA generated during year 1 using our TUTase knockdown cell lines, we plan on global microRNA profiling by Exiqon qPCR Arrays (UTHSC-H Quantitative Genomic/Microarray Core) as well as individual on both LIN28B-positive and LIN28A/LIN28B-double negative cell lines. The work on LIN28B cell lines where TUTase expression has been knocked out singly or in combination will define if LIN28B requires a TUTase to block let-7 maturation and if so, the identity of the responsible TUTase(s).

Task 6. Small molecule screen for modifiers of the LIN28B/let-7 pathway (Specific Aim 2)
We will continue to work on this Task as described in our initial application and SOW. Specifically, a pilot small molecule screen will be carried out by GCC John Dunn Screening Core. We will then validation candidate compounds and perform global microRNA profiling of cells treated with candidate compounds and controls by Exiqon qPCR Arrays (UTHSC-H Quantitative Genomic/Microarray Core). Lastly, we will seek to define experimentally the mode of action of those compounds that restore expression of the tumor suppressor let-7 microRNA family through potential inhibition of LIN28 or interacting TUTase(s).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Recent and provocative evidence implicates a novel form of post-transcriptional gene regulation involving 3' RNA uridylation mediated by Terminal Uridylyl Transferases (TUTases) as a critical gene regulator and driver of tumorigenesis. These data indicate that dysregulated TUTase activity alone and in concert with the onco-fetal LIN28/let-7 pathway are hallmarks of poor prognosis in multiple cancer types, including ovarian. Silent in almost all adult cells, the Lin28/let-7 pathway is implicated directly in cancer stem cell biology, disease recurrence, and resistance to ionizing radiation and several chemotherapies. The proposed research develops specific small molecule inhibitors of LIN28 and/or TUTases as a novel cancer therapy and addresses the roles of the TUTases in LIN28B-poitive and LIN28A/LIN28B-double negative breast cancer.

In year 1 of this research, we have developed numerous tools to interrogate LIN28 and TUTase function in ovarian cancer cells. Specifically, we have generated loss-of-function ovarian cancer cell lines for multiple genes (Negative control, LIN28A, LIN28B, ZCCHC6, and ZCCHC11) using two RNAi techniques, shRNA by lentiviral transduction or by transient transfection with siRNAs. In addition, we have also generated multiple gRNA lentiviral vectors (4 each for LIN28A, Lin28B, ZCCHC6, and ZCCHC11) for use with the CrispR/Cas9 system to generate ovarian cancer cell lines that have lost expression of these genes in their entirety. In parallel, we have also developed two cell-based systems (dual luciferase and antibiotic-resistance) to perform a pilot screen for small molecule inhibitors of LIN28 and/or their interacting TUTase(s) that restore expression of the tumor suppressor let-7 microRNA family.

In the long-term, our findings will advance understanding of how LIN28 and 3' RNA uridylation function in ovarian cancer and has widespread implications for other cancer types characterized by dysregulated expression of these genes. In addition, LIN28 and TUTase are attractive targets for therapeutic intervention in cancer and our work is an obligatory first step in defining small molecules that restore expression of the tumor suppressor let-7 microRNA family in LIN28-positive cancer. These molecules may lead to a novel class of cancercdrugs.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

No significant changes in the project or its direction have occurred. However, we note below minor differences in some techniques used in relation to what is written in the SOW to achieve the research tasks.

Changes in approach and reasons for change

Task 1. Acquisition of Human Ovarian Cancer Cell Lines (All Specific Aims, Months 1-2)
Several human ovarian cancer cell lines were obtained from a different source than initially listed in the SOW. This change occurred for two reasons: 1) cell line availability issues at Creative Bioarrays and 2) the temporary moratorium on providing cell lines from DTP/DCTD Tumor Repository of the NCI at the beginning of the funding period. Notably, OVK-18 was obtained from RIKEN Cell Bank, SK-OV-3 was obtained from ATCC, IGROV1 was a kind gift of Dr. Anil Sood, and COLO-704 was purchased from DSMZ-German Collection of Microorganisms and Cell Cultures.

Task 2/Task 3. Generation and Validation of TUTase and LIN28 Loss-of-Function Human Ovarian Cancer Cell Lines.
In our application, we proposed to use Cre-mediated recombination to generate shRNA knockdown cell lines for TUTases and LIN28. Unfortunately, we have not succeeded in generating the shRNA lines using this particular technique. Therefore, we used a standard lentiviral shRNA approach as we described previously to create the loss-of-function cell lines. This alternate method was explicitly included in our OCRP Pilot Award application [Aim 1A "Expected Results and Potential Caveats" Section (Page 4/Lines 22-25)]. Specifically, it was written: "If the proven doxycycline system for shRNA expression does not work for unexpected reasons, we will use standard lentiviral shRNA infections followed by puromycin selection to achieve knockdowns as we described previously[12]." In addition, we have utilized siRNAs as another loss-of-function RNAi technique (see above : 3. Accomplishments). The limitation of this RNAi method is that loss-of-function is transient. As an alternate method to generate stable loss-of-function cell lines to complement our already generated shRNA lines, we are developing handson experience with CrispR/Cas9 technology to generate stable cell lines where our genes of interest will be inactivated. The advantage of the CrispR/Cas9 method is that stable lines can be established that have one or more alleles mutated, resulting in loss-of-function. As such, it is possible to select clonally stable cell lines that are null for expression of our genes of interest in comparison to our stable shRNA lines where the expression of the targeted genes is reduced but not absent. As proof-of-principle, we have performed transient transfection with a dual Cas9/gRNA plasmid and have selected clones using limited dilution without selection. The gRNAs were designed to target exon 2 of LIN28A and LIN28B. For ZCCHC6 and ZCCHC11, the gRNA was designed against the exon containing the first two aspartates of the catalytic triad. Using this method, we have generated clonal cell lines where 1 or 2 alleles have been mutated for LIN28B. ZCCHC11, or ZCCHC6 (see above: 3. Accomplishments). To optimize further, we plan on introducing both Cas9 and specific gRNA for our genes of interest using lentiviral infection with antibiotic selection to improve the rate of generating null lines. The resulting cell lines will be validated as null at the protein level by Western.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. PRODUCTS:

Publications, conference papers, and presentations

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

RESEARCH MATERIALS

1) Lentiviral shRNA Vectors, Derived Lentivirus (Negative Control, LIN28A, LIN28B, ZCCHC6, and ZCCHC11), and Loss-of-Function Ovarian Cancer Cell lines form Lentiviral Transduction
2) Validated siRNAs to human LIN28A, LIN28B, ZCCHC6, ZCCHC11
3) Lentiiaral Cas9/gRNA Vectors and Derived Lentivrus (LIN28A, LIN28B, ZCCHC6, ZCCHC11)
4) Lentivaral Cas9 Vectors and Lentiviral Transduced Ovarian Cancer Cell Lines
5) Two distinct Let-7 Reporters (Dual lucifearase and TK-ZeoR) using various configurations of let-binding sites (based on human TRIM71, a well-characterized let-7 target gene)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	John P. Hagan
Project Role:	Principal Investigator
Researcher Identifier (ERA commons):	JPHAGAN
Nearest person month worked:	3
Contribution to Project:	Dr. Hagan was responsible for the overall execution of the project and has contributed to experimental design and troubleshooting.

Name:	Julien Balzeau
Project Role:	TEAL Postdoctoral Research Fellow
Researcher Identifier (ERA Commons):	JCBALZEAU
Nearest person month worked:	12
Contribution to Project:	Dr. Balzeau was responsible for the knockdown experiments performed in Aim 1.

Name:	Miriam Menezes
Project Role:	Postdoctoral Research Fellow
Researcher Identifier (ERA Commons):	MRMENEZES
Nearest person month worked:	2
Contribution to Project:	Dr. Menezes was primarily responsible for developing the cell-based screen for small molecules that restore let-7 expression in LIN28-positive ovarian cancer cell lines (LIN28A and LIN28B) outlined in Aim 2.

Name:	Siyu Cao
Project Role:	Postdoctoral Research Fellow
Researcher Identifier (ERA Commons):	CSIYU1
Nearest person month worked:	1
Contribution to Project:	Dr. Cao was responsible for a subset of the DNA subcloning that was required to develop the cell-based screen for small molecules that restore let-7 expression in LIN28-positive ovarian cancer cell lines (LIN28A and LIN28B) outlined in Aim 2.

Name:	Anil Sood. MD
Project Role:	Consultant
Researcher Identifier (ERA Commons):	AKSOOD
Nearest person month worked:	0 (24 hours @$125/hr)
Contribution to Project:	Dr. Sood has consulted on the project and provided the Igrov-1 ovarian cancer cell line. In addition, the Hagan research team now attends his weekly lab nmeetings that provide additional training in ovarian cancer research.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

Partnering Organization

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:

Nothing to Report.